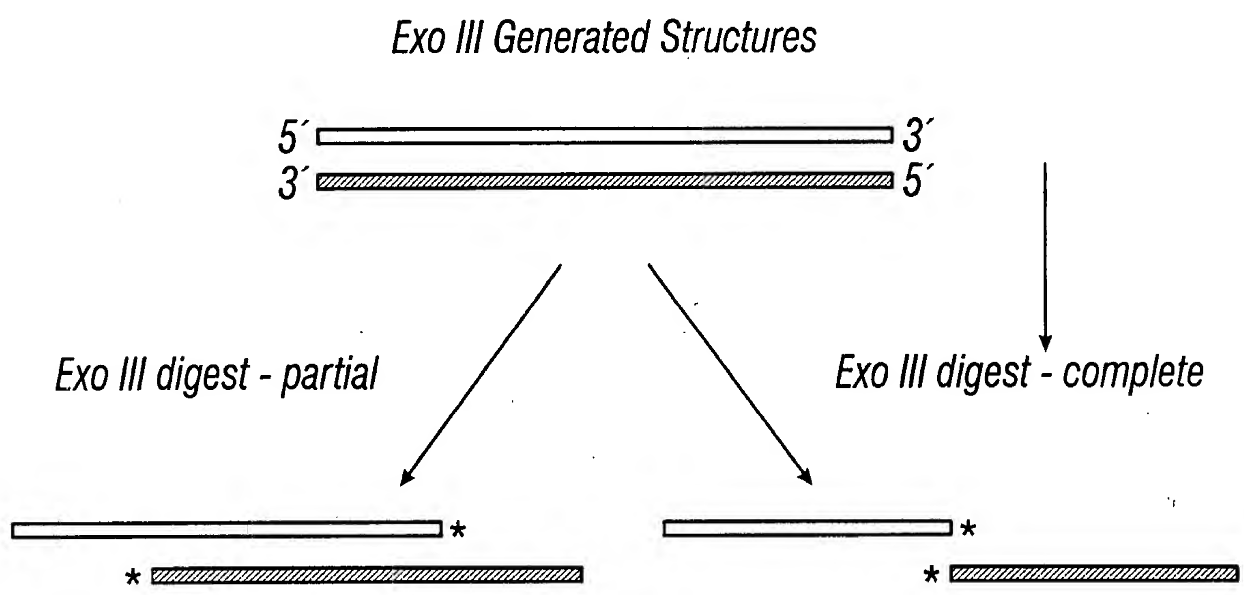

Exo III Generated Structures
5´
3´
3´
5´
Exo III digest - partial
Exo III digest - complete
*
*
*
*

FIG. 1

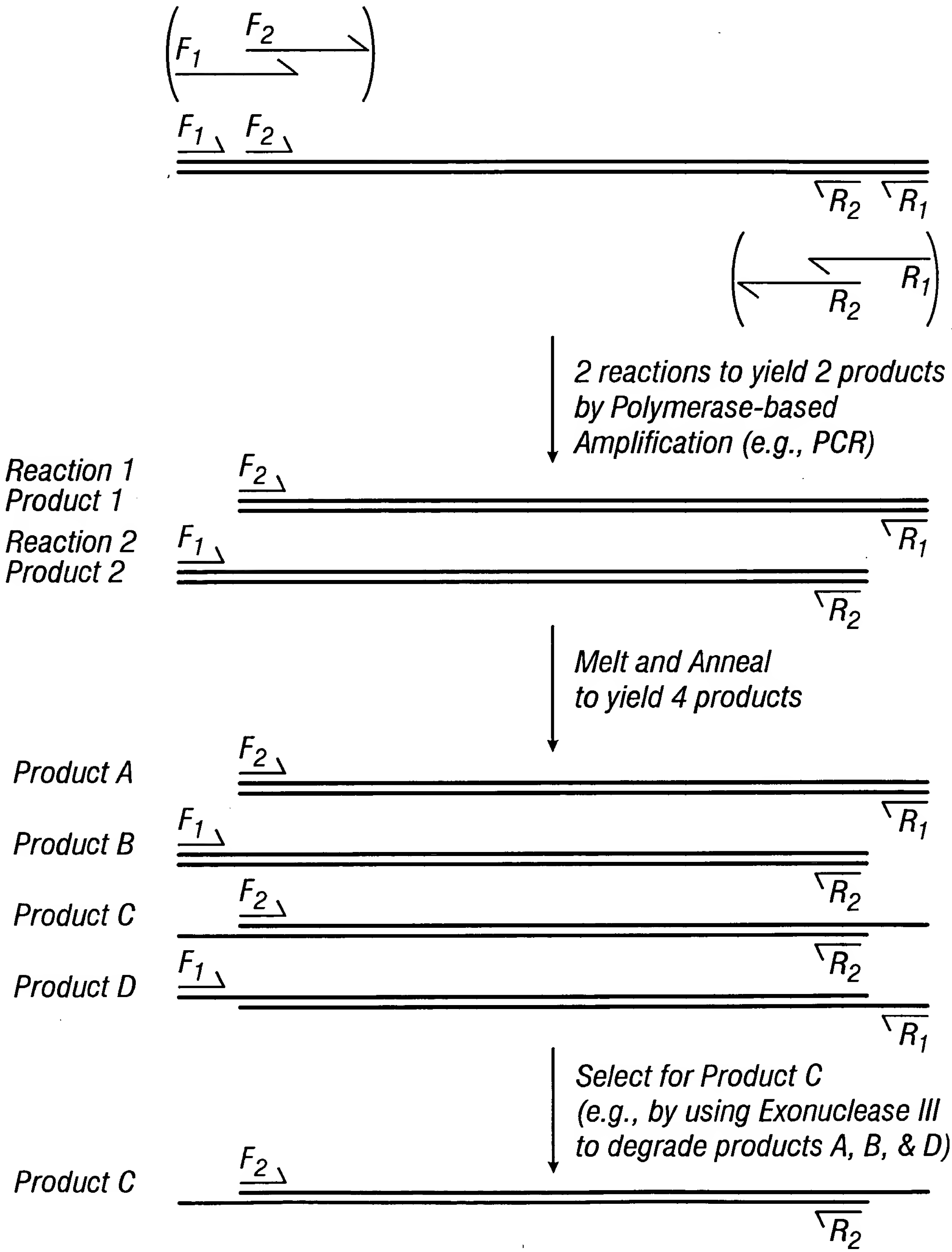

F1
F2
F1
F2
R2
R1
R1
R2
2 reactions to yield 2 products by Polymerase-based Amplification (e.g., PCR)
Reaction 1
Product 1
F2
R1
Reaction 2
Product 2
F1
R2
Melt and Anneal to yield 4 products
Product A
F2
R1
Product B
F1
R2
Product C
F2
R2
Product D
F1
R1
Select for Product C (e.g., by using Exonuclease III to degrade products A, B, & D)
Product C
F2
R2

FIG. 2

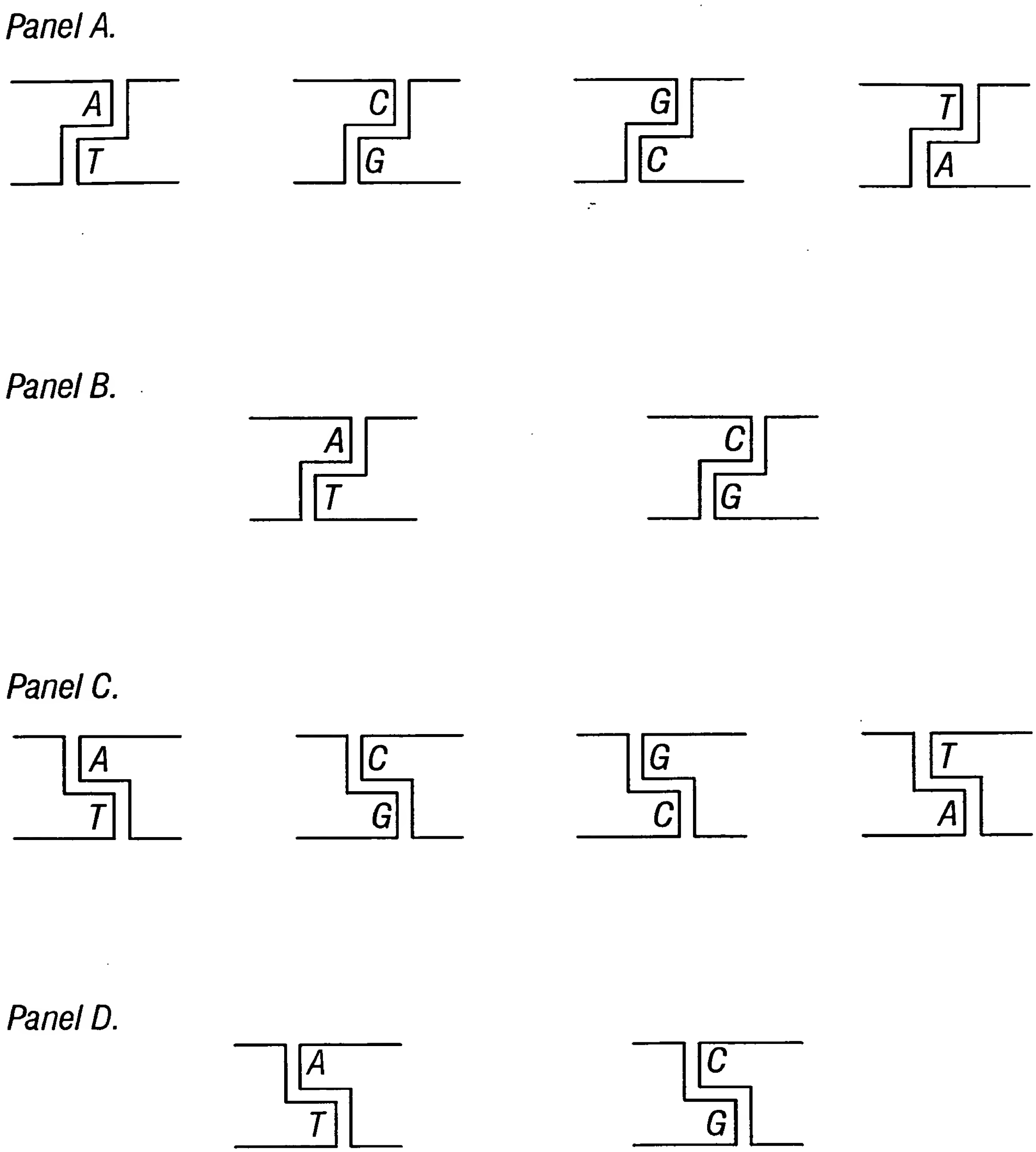


FIG. 3

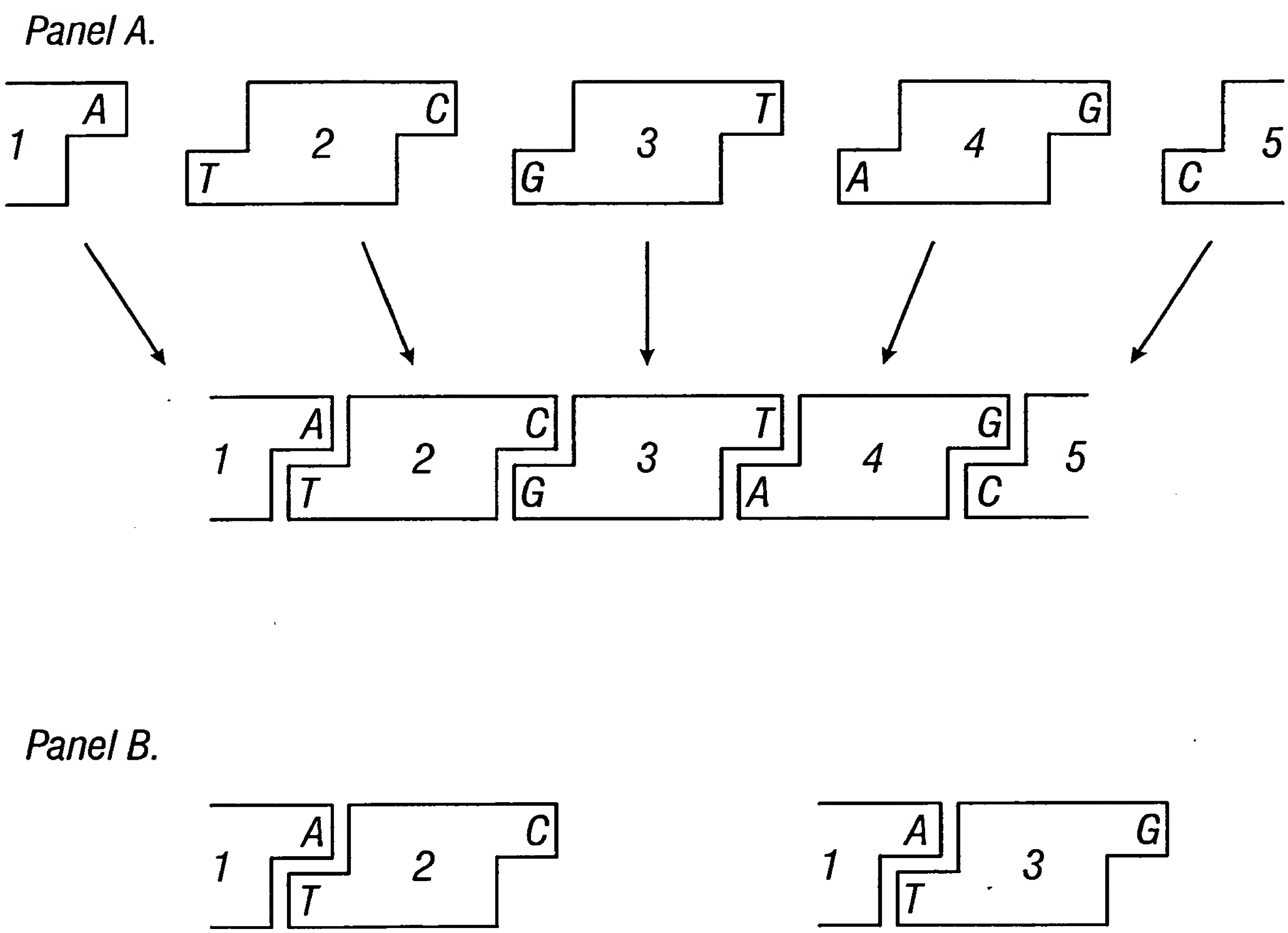


FIG. 4A

Panel C.

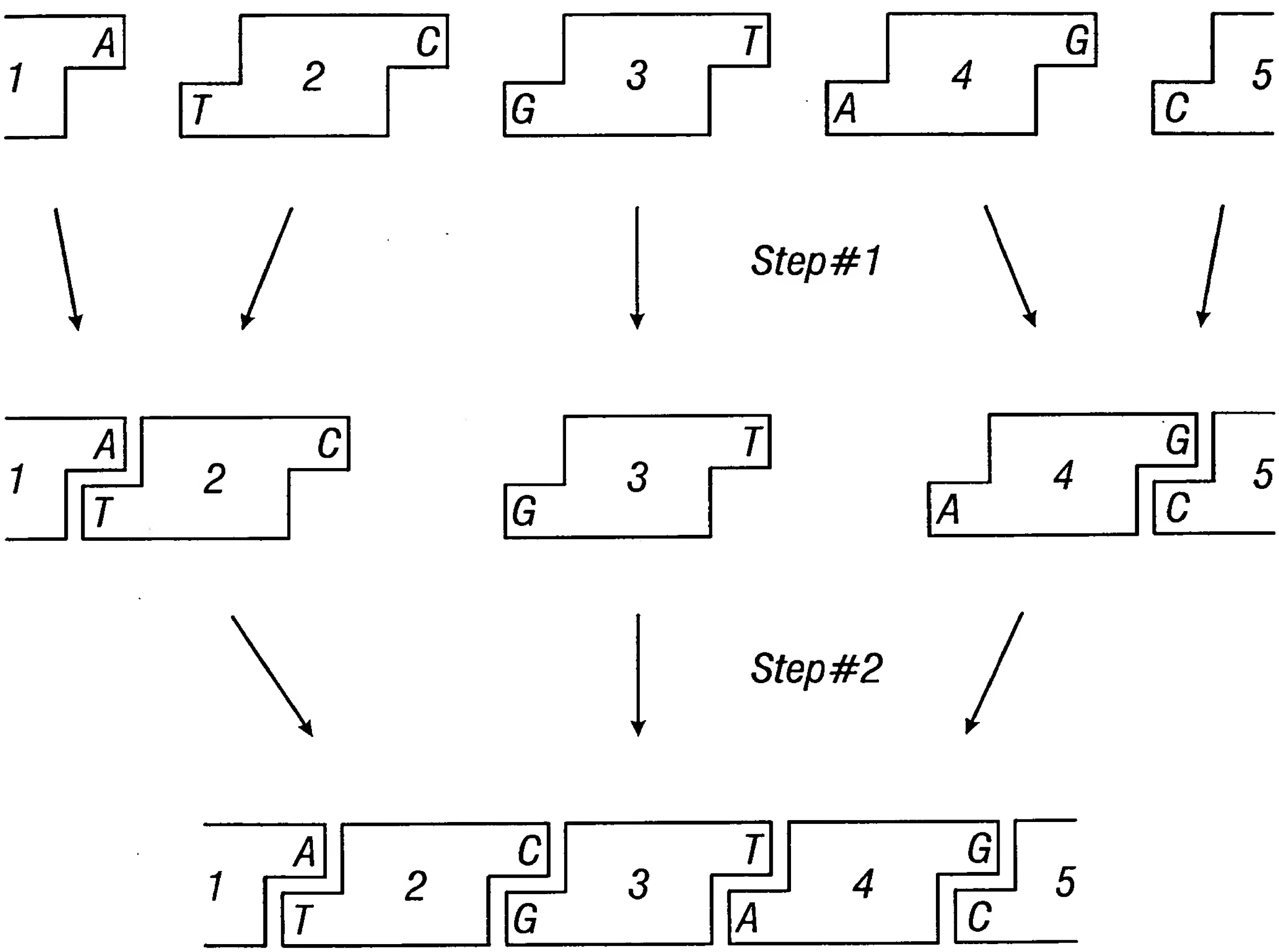


FIG. 4B

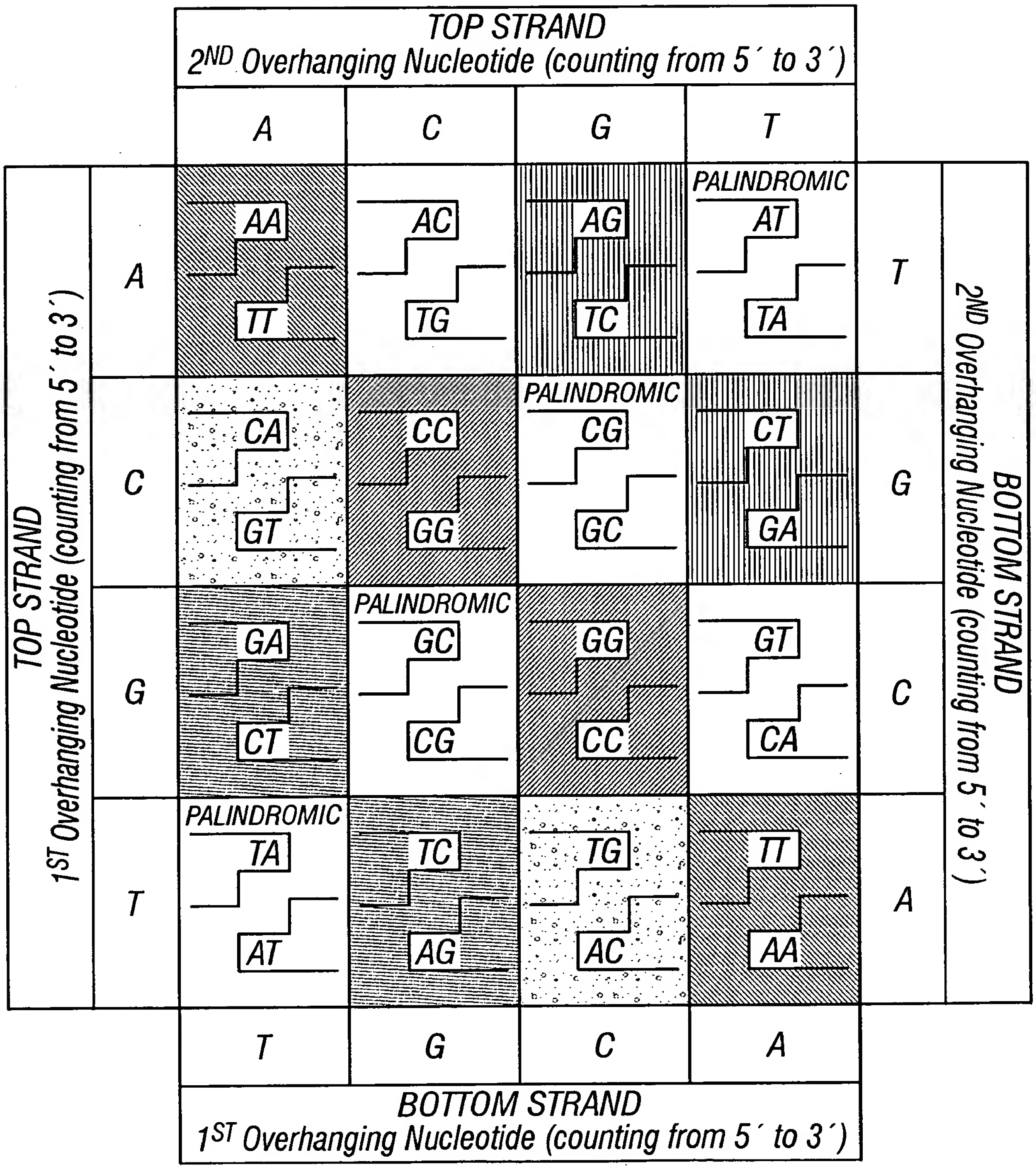
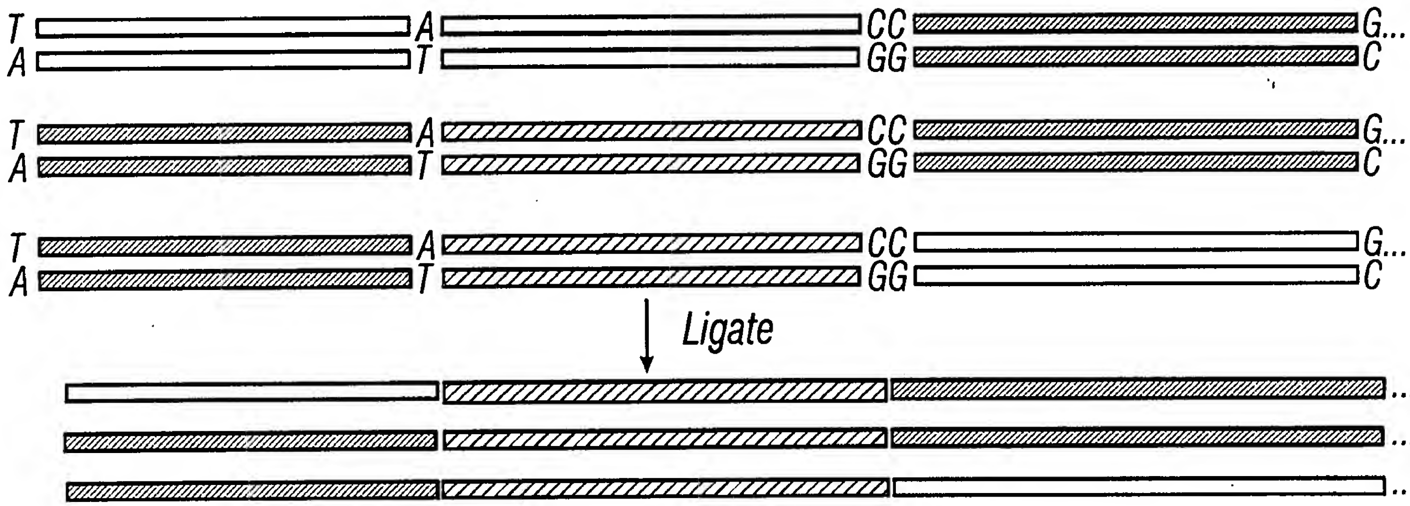

TOP STRAND
2ND Overhanging Nucleotide (counting from 5´ to 3´)
A
C
G
T
TOP STRAND
1ST Overhanging Nucleotide (counting from 5´ to 3´)
A
C
G
T
AA
TT
AC
TG
AG
TC
PALINDROMIC
AT
TA
CA
GT
CC
GG
PALINDROMIC
CG
GC
CT
GA
GA
CT
PALINDROMIC
GC
CG
GG
CC
GT
CA
PALINDROMIC
TA
AT
TC
AG
TG
AC
TT
AA
T
G
C
A
BOTTOM STRAND
2ND Overhanging Nucleotide (counting from 5´ to 3´)
T
G
C
A
BOTTOM STRAND
1ST Overhanging Nucleotide (counting from 5´ to 3´)

FIG. 5

Select for full length

```
          1                                                     50
 124-2d AATGGACAAG AAACGTGTCC GTGTGTACAA CGCGGAGATG GCCTATGTCG
  12412 ~ATGGAGAAA CACCGCGTAG AAGTTCTCGG TTCGGAGATG GCCTACATCG
 124-1d ACACGACAAG CGCTACATCG AGGTGCTGGG TAAGCGAATG GCCTATGTCG
  myco1 CGGGCAGCCG AAGTACCTAG AAATCGCCGG GAAGCGCATG GCGTATATCG
     b3 CTACCCAAAA TTTCGGCGGT CGGTCTTCGG CCGCGAGATG GCGTACGTGG
     b1 GCATCCGAGA AAGCGGATCG CCGTGCTCGA TTCGGAGATG AGCTACGTCG
  15112 ~ATGCCAGCG ATTGAGCTAT TGGATTCGTT CATGAACTAC CGCGACACGG
  rhod2 CCCCCATTAT GTGGAAGTCC TGGGCGAGCG TATGCACTAC GTCGATGTTG
Consensus---------- ---------- ---------- ---------- --- A----G
```

8 + 8 + 8 + $n = 144$ d.s oligos



$8^{18} = 2x10^{16}$ Reassembled Gene Variants

FIG. 6A

```
                                                     100
ACACGGGCCA GGGTGATTCC GTTCTGTTTC TTCACGGCAA CCCGACGTCG
ACGTGGGAGA GGGCGACCCG ATCGTGTTCC TCCACGGAAA TCCCACGTCG
AGATGGGCGA GGGTGATCCC ATCATTTTCC AACACGGCAA TCCGACCTCA
ACGAAGGCAA GGGTGACGCC ATCGTCTTTC AGCACGGCAA CCCCACGTCG
AAGTGGGACG GGGCGACCCC ATCGTACTCT TGCACGGCAA CCCCACCTCG
ATACCGGCGA GGGAGCGCCG ATCGTGTTCC TTCACGGCAA CCCGACTTCC
GCGTCGGCGA T...CTTCCC GTCGTGTTCC TGCACGGCAA CCCCACGTCG
GACCGCGGGA TGGCACGCCT GTGCTGTTCC TGCACGGTAA CCCGACCTCG
------G--- --------C- -T--T--T-- --CACGG-AA -CC-AC-TC-
```

FIG. 6B

Represents 15% of gene

```
                                                     150
TCGTATCTGT GGAGGGGCGT AATGCCTTTT GTGACGGACG TCGCCCGATG
TCGTACCTGT GGCGGAACGT GATTCCCCAC GTTGCCGGCT TGGGACGCTG
TCGTACCTGT GGCGCAACAT CATGCCCCAT GTGCAACAGC TCGGTCGCTG
TCTTACTTGT GGCGCAACAT CATGCCGCAC TTGGAAGGGC TGGGCCGGCT
TCGTACCTCT GGCGCAACGT GTTGCCGCAC CTGGCGCCGT TAGGCCGCTG
TCCTATCTTT GGCGCAACAT CATCCCCTAT CTCGCGGATC ACGGCAGATG
TCTCACGTCT GGCGCAACGT GATCCCGCAC GTCGCTGGCC AGCACCGGTG
TCCTACCTGT GGCGCAACAT CATCCCGCAT GTAGCACCGA GTCATCGGTG
TC--A--T-T GG-G---C-T --T-CC---- -T-------- ------G---
```

FIG. 6C

```
           NcoI                                                         CCGT
150am13_00 cATGATGCACG GCGATATTTC ATCGAGCAAT GACACGGTCG GCGTTGCCGT
150AM7_001 cATGCATCACG GCGACATTTC ATCGAGCAAT GACACGGTCG GCGTTGCCGT
431am7_002 cATGAGACACG GAGATATCTC CAGCAGCAAC GATTGCGTGG GCGTGGCCGT
                                                      GAG GT
150am13_00  CGTGAACTAC AAGATGCCTC GCCTTCATAC CAAGGCGGAG GTTTTAGCGA
150AM7_001  CGTGAACTAC AAGATGCCGC GGCTTCACAC CAAGGCTGAG GTGCTGGCCA
431am7_002  CGTGAACTAC AAGATGCCGC GGCTGCATAC CCGCGCGGAG GTGATGGAGA
                                                          CGG
150am13_00  ACGCCAGAAA GATCGGCGAG ATGATCGTCG GCATGAAGAC CGGCCTGCCC
150AM7_001  ACTGCCGCAA GATCGCCGAC ATGCTGGTCG GCATGAAGAG CGGCCTGCCG
431am7_002  ACGCCCGCAA GATCGCCGAC ATGGTCGTGG GCATGAAGCG CGGCCTGCCC
                                                 CCACG
150am13_00  GGAATGGATC TGGTGATCTT CCCGGAATAT TCGACCCACG GCATCATGTA
150AM7_001  GGAATGGATC TGGTGATCTT CCCGGAATAT TCCACCCACG GCATCATGTA
431am7_002  GGCATGGACC TGGTCATCTT CCCCGAGTAC TCCACCCACG GCATCATGTA
                                                      CCC GG
150am13_00  CGACTCCAAG GAAATGTACG ATACCGCGTC CGTCGTGCCC GGCGAGGAGA
150AM7_001  CGACTCCAAG GAGATGTACG ACACGGCGTC GACGGTGCCG GGTGAAGAGA
431am7_002  CGACGCCAAG GAAATGTACG AAACCGCTTC GGCCATTCCG GGCGAAGAGA
                                                        G GGG
150am13_00  CCGAGATTTT TGCCGAAGCC TGCCGCAAGG CGAAAGTCTG GGGCGTGTTC
150AM7_001  CCGAGATTTT CGCCGAGGCC TGCCGCAAGG CCAAGGTCTG GGGCGTGTTC
431am7_002  CTGCTGTGTT CGCCGACGCC TGCCGCAAGG CCAACGTATG GGGCGTGTTT
```

FIG. 7A

```
                                                         AAAG C
150am13_00   TCGCTCACCG GCGAACGTCA CGAGGAACAT CCGAAGAAGG CGCCCTACAA
150AM7_001   TCGCTGACCG GCGAGCGCCA CGAGGAGCAT CCCAATAAAG CGCCGTACAA
431am7_002   TCGCTGACGG GCGAGCGCCA CGAAGAGCAC CCGAACAAGG CGCCGTACAA
                                                          CAG AA
150am13_00   CACGCTGATC CTGATGAACG ACAAGGGCGA GGTGGTCCAG AAATACCGCA
150AM7_001   CACCCTGATC CTGATGAACG ACAAGGGTGA AGTCGTTCAG AAATATCGCA
431am7_002   CACGCTCATC CTGATGAACA ACAAGGGCGA GATCGTGCAG AAGTACCGCA
                                             GGTA
150am13_00   AGATCATGCC GTGGGTTCCG ATCGAGGGCT GGTACCCCGG CAACTGCACC
150AM7_001   AGATCATGCC GTGGGTGCCG ATCGAAGGCT GGTATCCCGG CAACTGCACG
431am7_002   AGATCATGCC CTGGGTGCCG ATCGAAGGCT GGTATCCGGG CGATTGCACC
                                        TGAAG
150am13_00   TACGTCTCCG ACGGGCCGAA GGGCATGAAG GTTTCGCTGA TCATCTGCGA
150AM7_001   TACGTCTCCG AAGGCCCGAA GGGCATGAAG ATGTCGCTGA TCATCTGCGA
431am7_002   TATGTGTCGG AAGGCCCCAA GGGACTGAAG ATCAGCCTCA TCATCTGCGA
                                  TCTGGCG
150am13_00   TGACGGCAAC TATCCGGAAA TCTGGCGCGA CTGCGCCATG AAGGGCGCCG
150AM7_001   CGACGGCAAC TACCCGGAAA TCTGGCGTGA CTGCGCGATG AAGGGCGCCG
431am7_002   CGACGGCAAT TACCCCGAGA TCTGGCGCGA TTGCGCCATG CGCGGCGCCG
                             CCAG
150am13_00   AGCTGATCGT GCGCTGCCAG GGCTACATGT ATCCGGCCAA GGACCAGCAG
150AM7_001   AACTGATCAT CCGCTGCCAG GGCTACATGT ATCCCGCCAA GGATCAGCAG
431am7_002   AGCTGATCGT GCGTTGCCAG GGATACATGT ACCCGGCCAA GGACCAGCAG
```

FIG. 7B

```
                                 GC
150am13_00   GTCATCATGG CGAAGGCGAT GGCGTGGGCG AATAATTGTT ACGTCGCGGT
150AM7_001   GTGCTGATGG CGAAAGCAAT GGCCTGGGCC AACAACGTTT ATGTCGCGGT
431am7_002   GTCATGGTGT CCAAGGCCAT GGCGTGGATG AACAACGTCT ACGTGGCGGT
                           GGGCTTCG
150am13_00   TTCCAATGCC GCGGGCTTCG ATGGCGTCTA TTCGTATTTC GGCCACTCGG
150AM7_001   CGCCAATGCC TCGGGCTTCG ACGGCGTCTA CTCGTATTTC GGCCATTCGG
431am7_002   GGCCAATGCC GCGGGCTTCG ACGGCGTGTA TTCCTACTTC GGCCATTCGG
               TTCGA
150am13_00   CGATCATCGG CTTCGATGGC CGCACGCTCG GCGAATGCGG CGAGGAAGAA
150AM7_001   CGATCATCGG CTTCGACGGC CGTACCCTCG GCGAATGCGG CGAGGAGGAT
431am7_002   CCATCATCGG CTTCGACGGC CGCACGCTGG GCGAATGCGG TGAAGAAGAC
            C AGTA
150am13_00   TACGGCATCC AGTATGCCCA GCTTTCGAAG ATGCTGATCC GCGACGCCCG
150AM7_001   TATGGCATCC AGTATGCCGC CATCTCCAAG TCGCTGATCC GCGACGCGCG
431am7_002   ATGGGCGTGC AGTACGCCGA GCTCTCCACC AGCCTGATCC GCGACGCGCG
               CAATC
150am13_00   CCGCACCGGA CAATCGGAAA ACCATCTCTT CAAGCTGGTG CATCGTGGCT
150AM7_001   CCGCACCGGC CAATCGGAAA ACCATCTCTT CAAGCTGGTG CACCGTGGCT
431am7_002   CAAGAACATG CAGTCGCAGA ACCACTTGTT CAAGCTGGTG CACCGCGGCT
               GATCAA
150am13_00   ACACCGGGTT GATCAACTCC GGCGAGGGCG ACCGCGGTCT CGCGGCCTGT
150AM7_001   ACACCGGCAT GATCAATTCC GGCGAGGGCG ACCGCGGTGT CGCGGCTTGC
431am7_002   ACACCGGCAA GATCAATTCC GGCGAAGAGG CCACCGGCGT CGCGGCATGC
```

FIG. 7C

```
                  TTA
150am13_00   CCTTATGAGT TCTACAACAA ATGGATCGCC GATCCGGAAG GCACCCGCGA
150AM7_001   CCGTATGATT TCTATTCGAA ATGGATCGCC GATCCCGAGG GTACACGCGA
431am7_002   CCGTACAACT TCTACGCCAA CTGGATCAAC GATCCGGAGG GCACGCGCAA
              ATGGT
150am13_00   AATGGTCGAG TCCTTTACCC GGCCGACGGT GGGAACCGAT GAAGCGCCCA
150AM7_001   GATGGTGGAA TCCTTCACGC GTCCGACGGT GGGTGTGGAG GAATGCCCGA
431am7_002   GATGGTCGAA TCCTTCACCC GGTCCACCGT GGGCACGCCG GAGTGCCCCA
             TCGAG
150am13_00   TCGAAGGCAT CCCGAACAAG GTCGCGGTGC ACCGCTGA   aagct
150AM7_001   TCGAGGGCAT TCCGAACAAG GCCACCACGC ACCGCTGA   aagct
431am7_002   TGGACGGCAT CCCCAACGAG GACGCCAAGC ACCGCTAG   aagct
                                                          HindIII
```

FIG. 7D

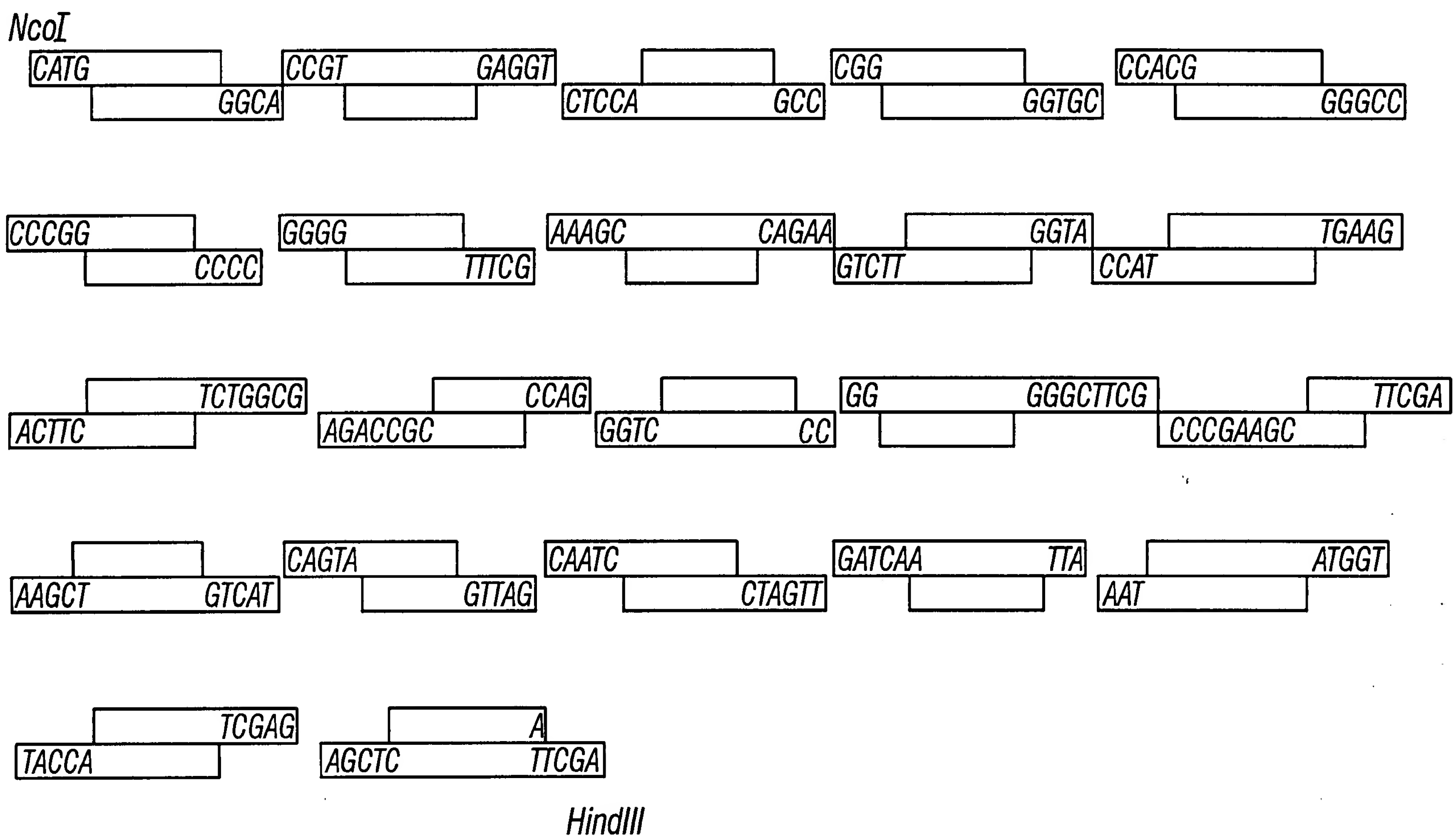

NcoI
CATG
GGCA
CCGT
GAGGT
CTCCA
GCC
CGG
GGTGC
CCACG
GGGCC
CCCGG
CCCC
GGGG
TTTCG
AAAGC
CAGAA
GTCTT
GGTA
TGAAG
CCAT
TCTGGCG
ACTTC
CCAG
AGACCGC
GGTC
CC
GG
GGGCTTCG
CCCGAAGC
TTCGA
AAGCT
GTCAT
CAGTA
GTTAG
CAATC
CTAGTT
GATCAA
TTA
ATGGT
AAT
TCGAG
TACCA
A
AGCTC
TTCGA
HindIII

FIG. 8

FIG. 9

Gap Ligation

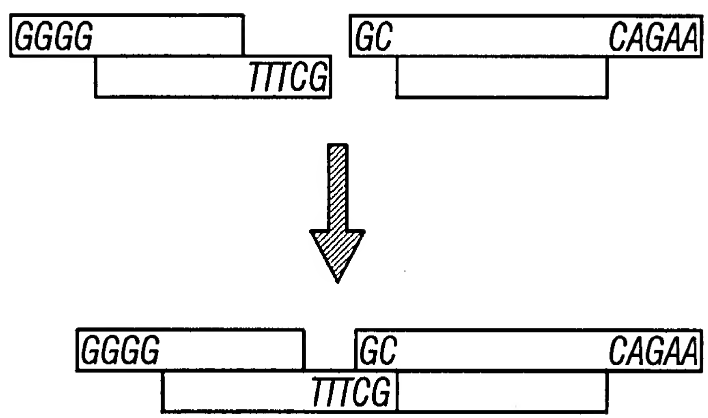


FIG. 10

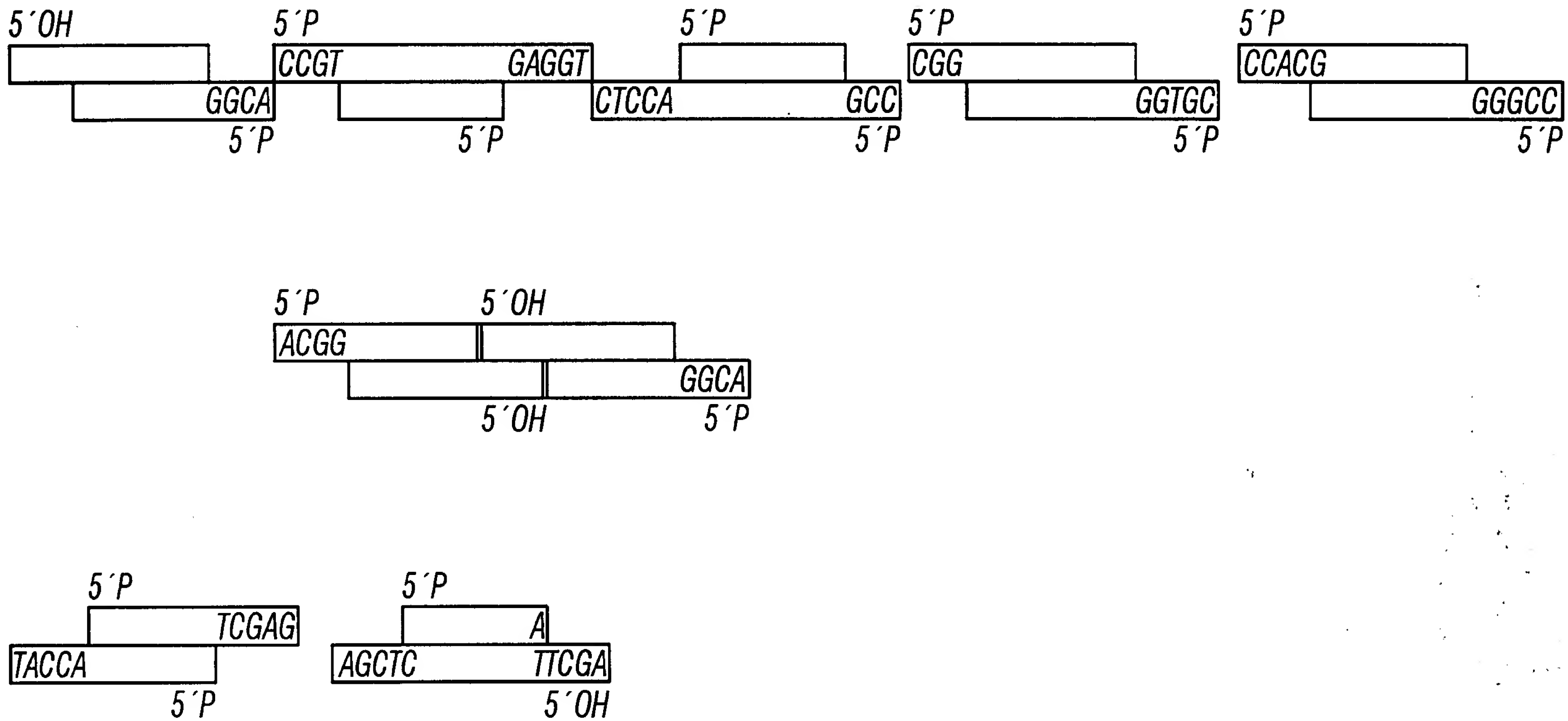

5´OH
5´P
CCGT
GAGGT
GGCA
5´P
5´P
CTCCA
GCC
5´P
5´P
CGG
GGTGC
5´P
5´P
CCACG
GGGCC
5´P
5´P
5´OH
ACGG
GGCA
5´OH
5´P
5´P
TCGAG
TACCA
5´P
5´P
A
AGCTC
TTCGA
5´OH

FIG. 11

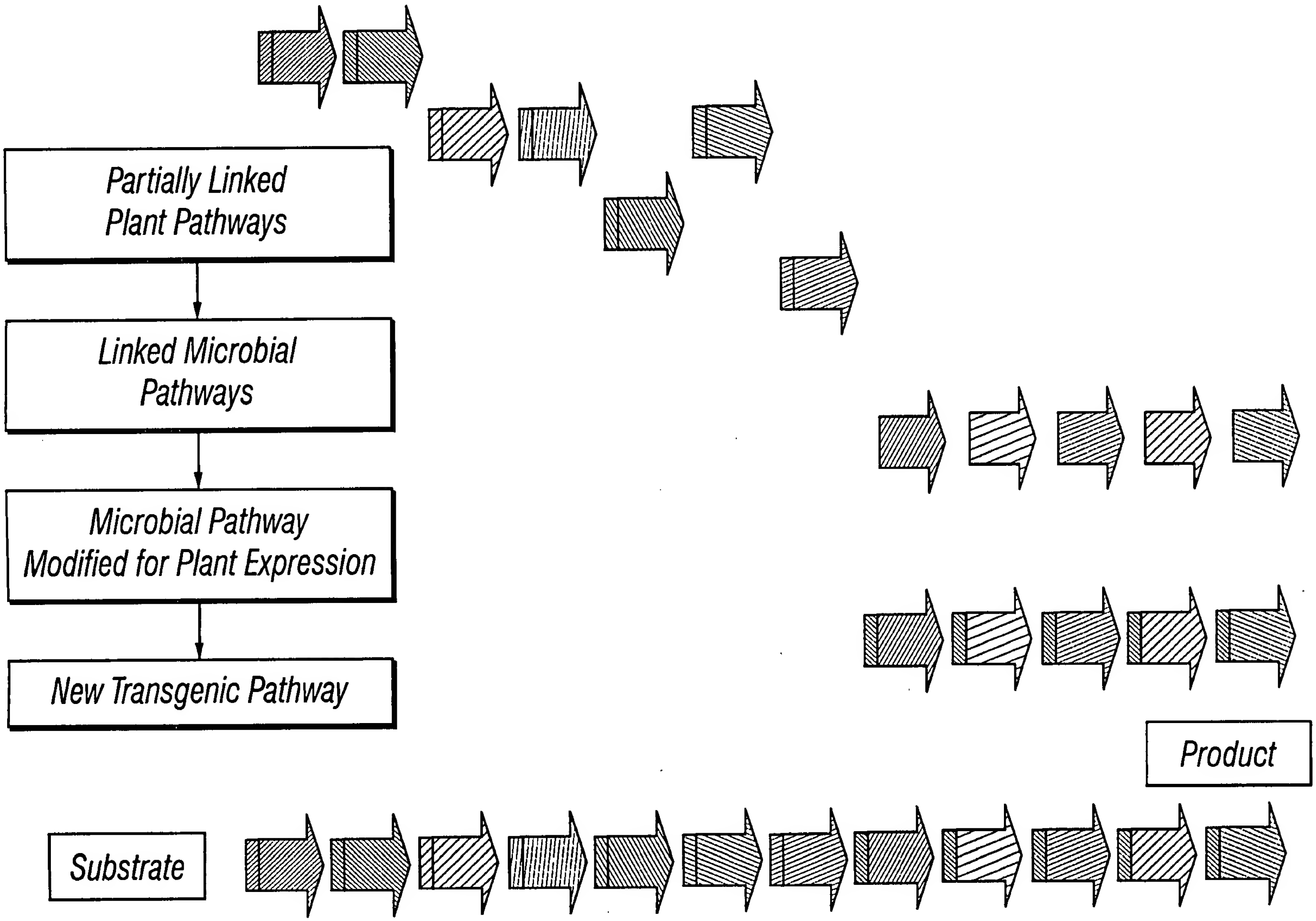

Partially Linked Plant Pathways
Linked Microbial Pathways
Microbial Pathway Modified for Plant Expression
New Transgenic Pathway
Product
Substrate

FIG. 12

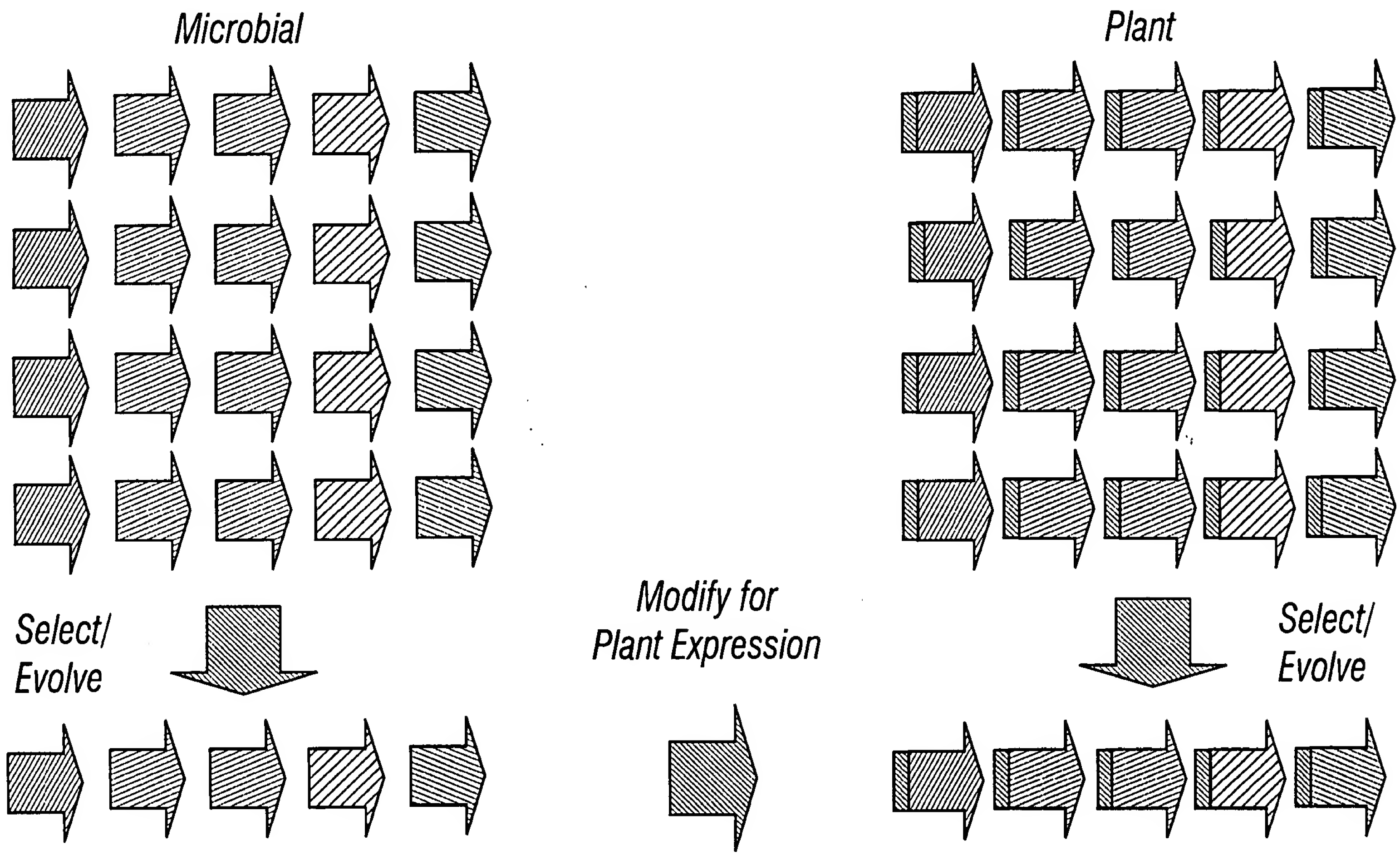

Microbial
Plant
Select/
Evolve
Modify for
Plant Expression
Select/
Evolve

FIG. 13

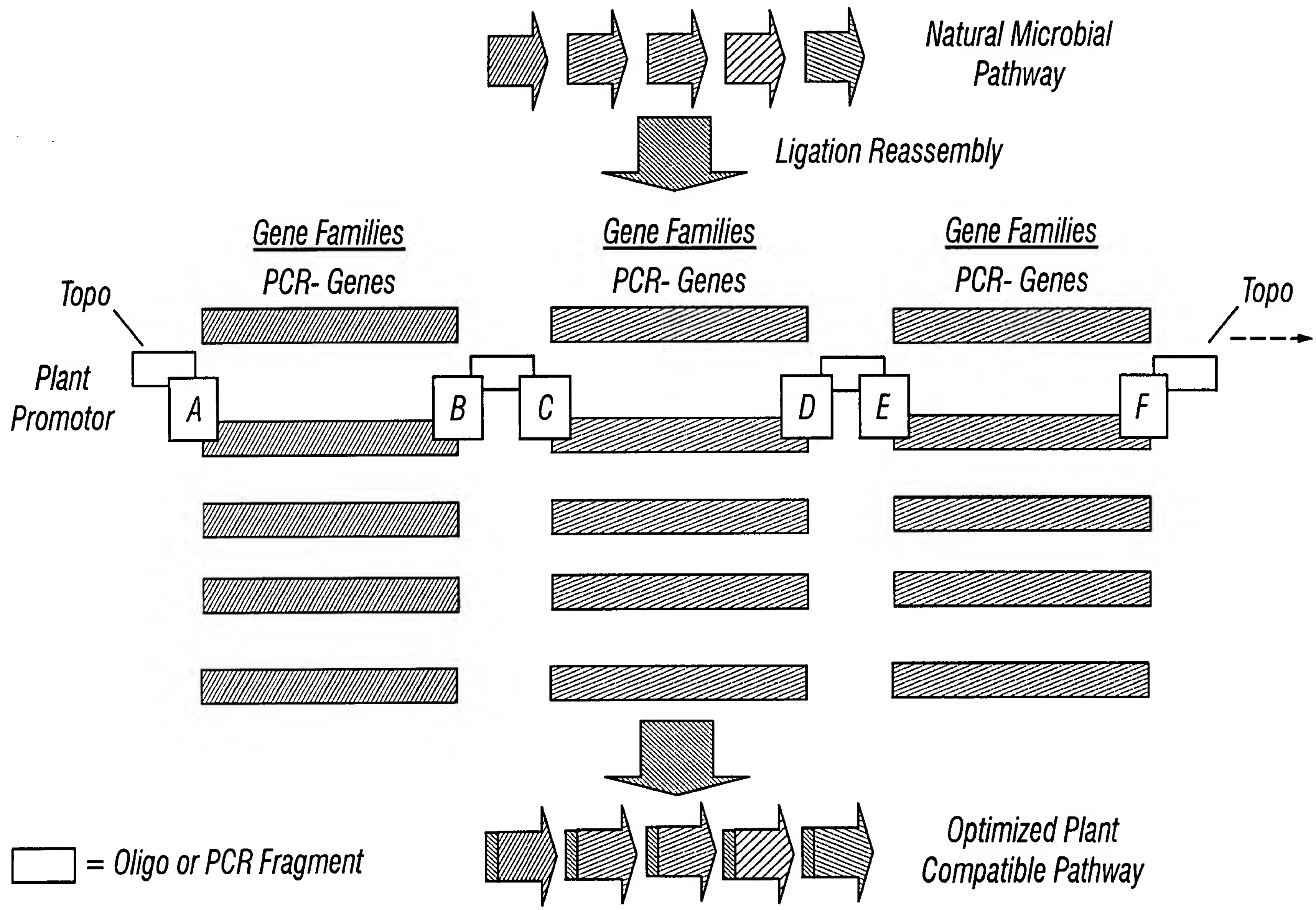
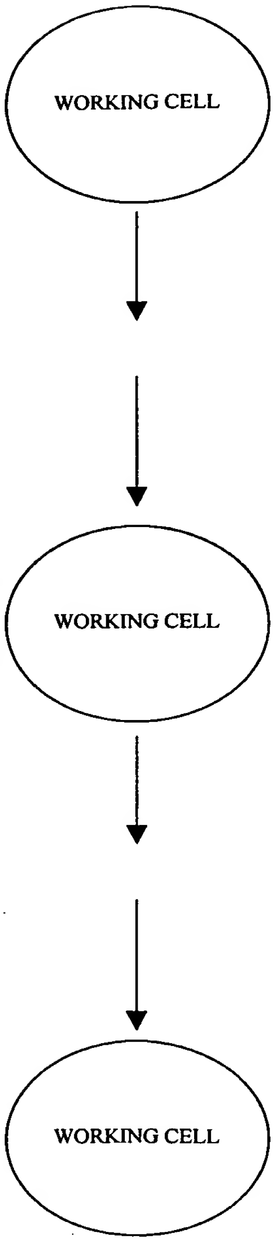

Natural Microbial Pathway
Ligation Reassembly
Gene Families
PCR- Genes
Gene Families
PCR- Genes
Gene Families
PCR- Genes
Topo
Plant Promotor
A
B
C
D
E
F
Topo
Optimized Plant Compatible Pathway
= Oligo or PCR Fragment

FIG. 14

Fig. 15. HOLISTIC ENGINEERING OF DIFFERENTIALLY ACTIVATABLE STACKED TRAITS IN NOVELTRANSGENIC PLANTS USING DIRECTED EVOLUTION AND WHOLE CELL MONITORING



1.) Whole cell monitoring (can include all detectable functions and physical parameters). Examples include morphology, behavior, growth, responsiveness to stimuli (e.g., antibiotics, different environment, etc.). May also include all measurable molecules, including nucleic acids, proteins, carbohydrates, proteoglycans, glycoproteins, and lipids.

2.) Introduce stacked traits (can include selectively and differentially activatable traits).

3.) Whole cell monitoring.

4.) Data compilation, processing, analysis, determination of correlations among measurable parameters.

5.) Further genetic manipulations

6.) Optionally repeat any or all steps, 1 - 3.

7.) Optionally subject to any or all steps, 3-6.

Fig. 16. Differential Activation of Selected Traits Can Be Achieved by Adjusting and Controlling the Environment of the Traits.

For example, in one aspect, stacked traits can be comprised of genetically introduced enzymes. Because the stacked enzymes have different activity profile (including reaction specificities and reaction requirements) they can be selectively and differentially activated by adjusting the environment to which they are exposed.

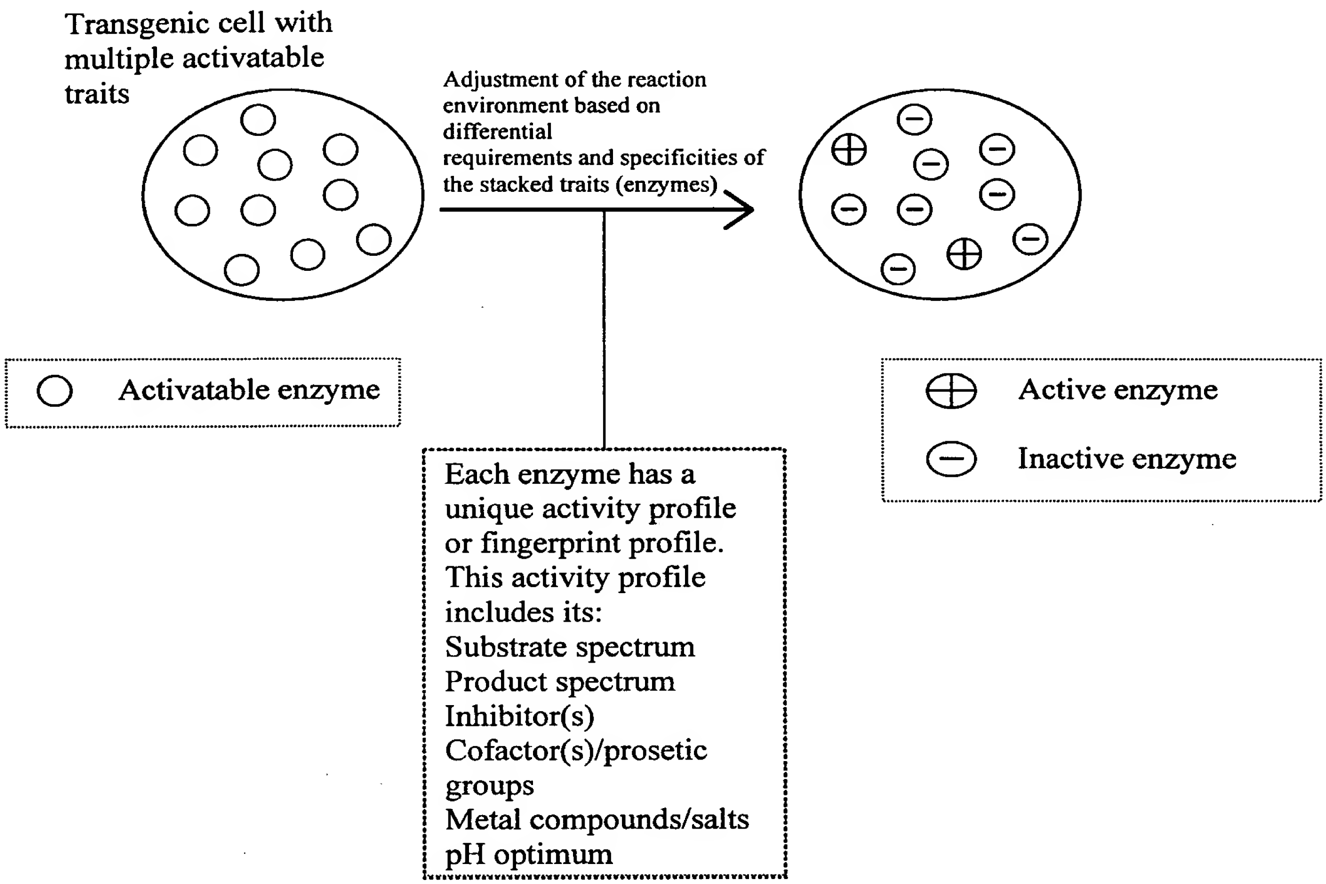


Fig. 17. **Harvesting, Processing, Storage**

Differentially activated and/or selected enzymes respond to the environments of harvesting, processing and storage to activate environmentally action specific promoters.

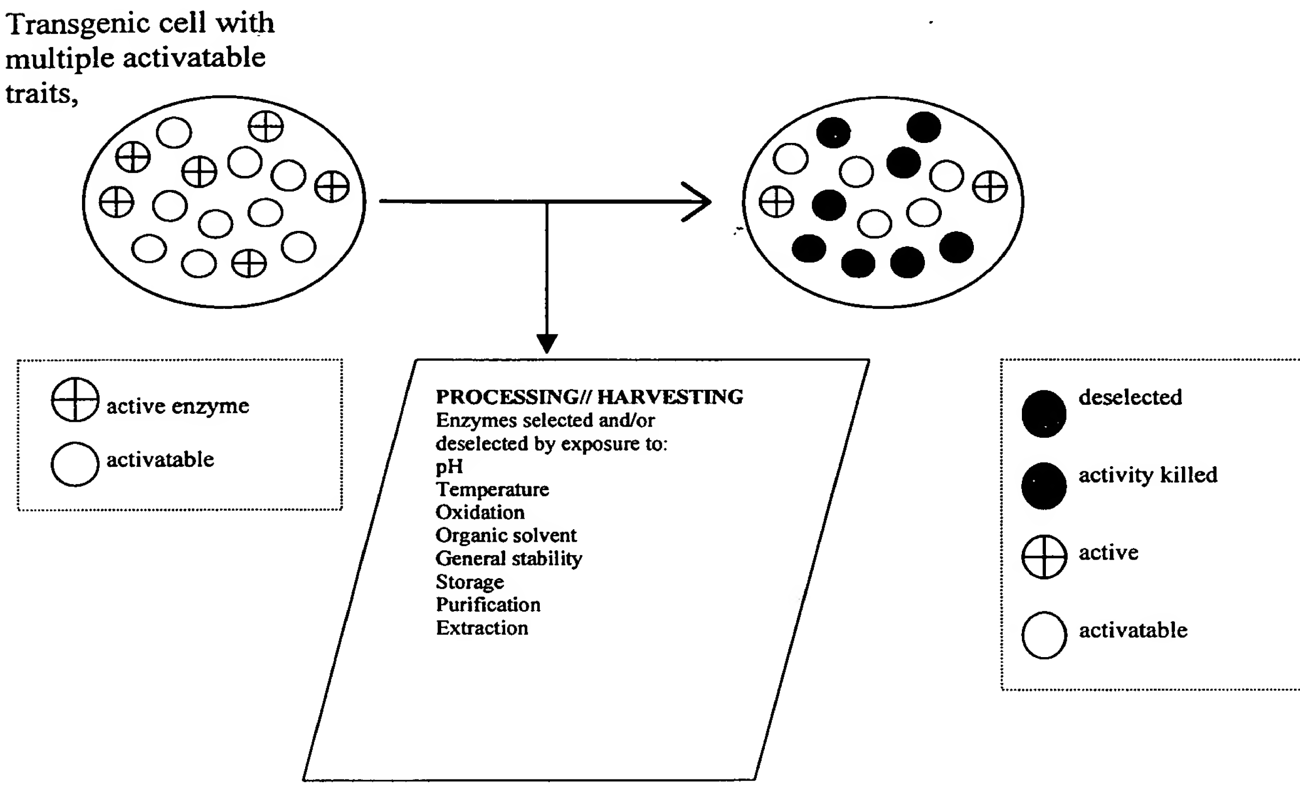
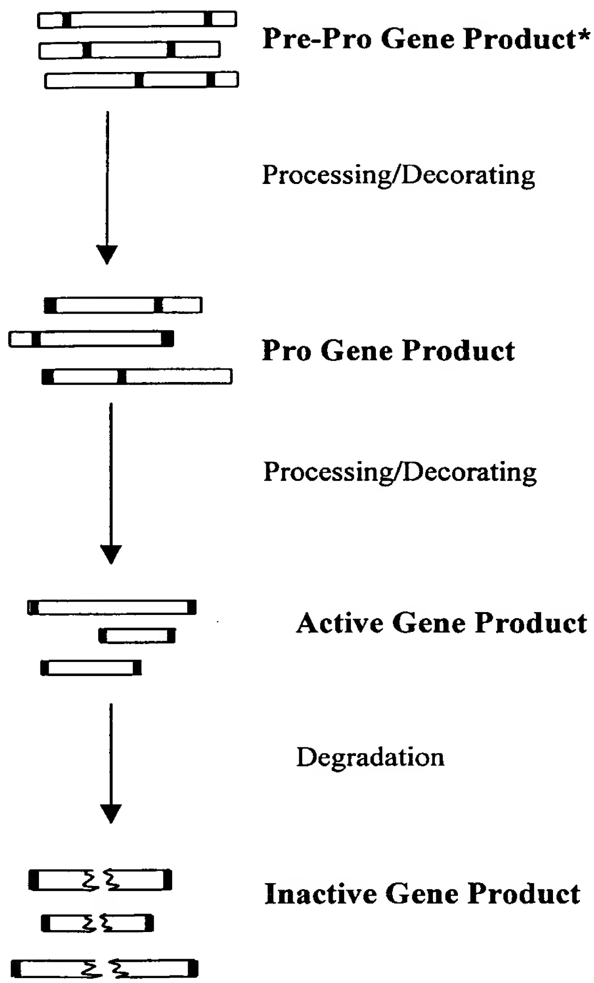


Fig. 18. Processing



* An example of a Gene Product might be a protein. Through processing/decorating the protein changes forms, eventually becoming active. It is at this point that specific traits can be expressed differentially.

Fig. 19. Cellular Mutagenesis.

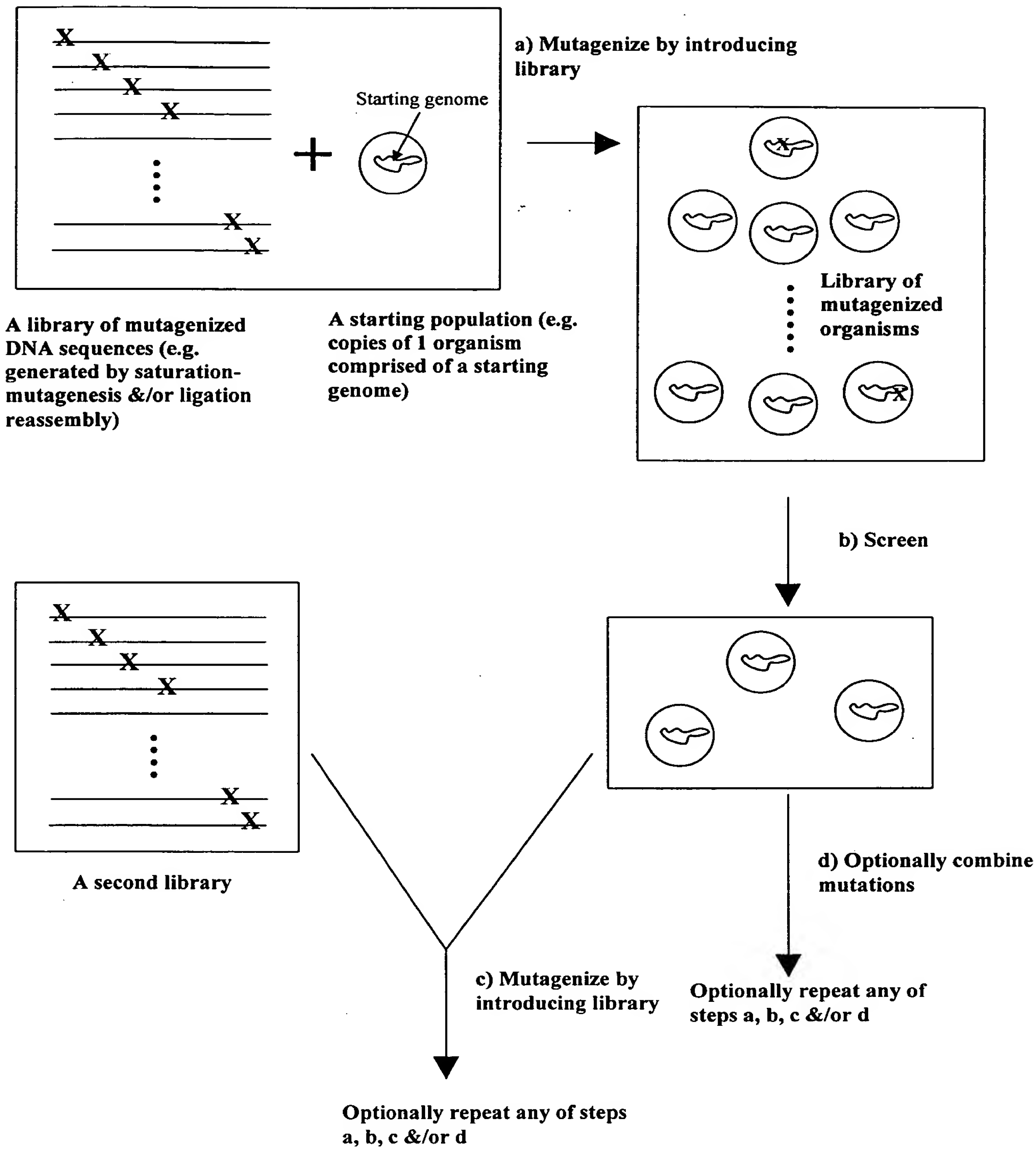


Fig. 20. Differential Activation of Selected Precursor (Inactive) Gene Products

Differential activation of selected precursor (inactive) gene products by controlling the post-translational modifications that differentially transform selected molecules from inactive precursor form to active form. Deselection of particular molecules can also be achieved by degradation (ex. By proteoclytic cleavage).

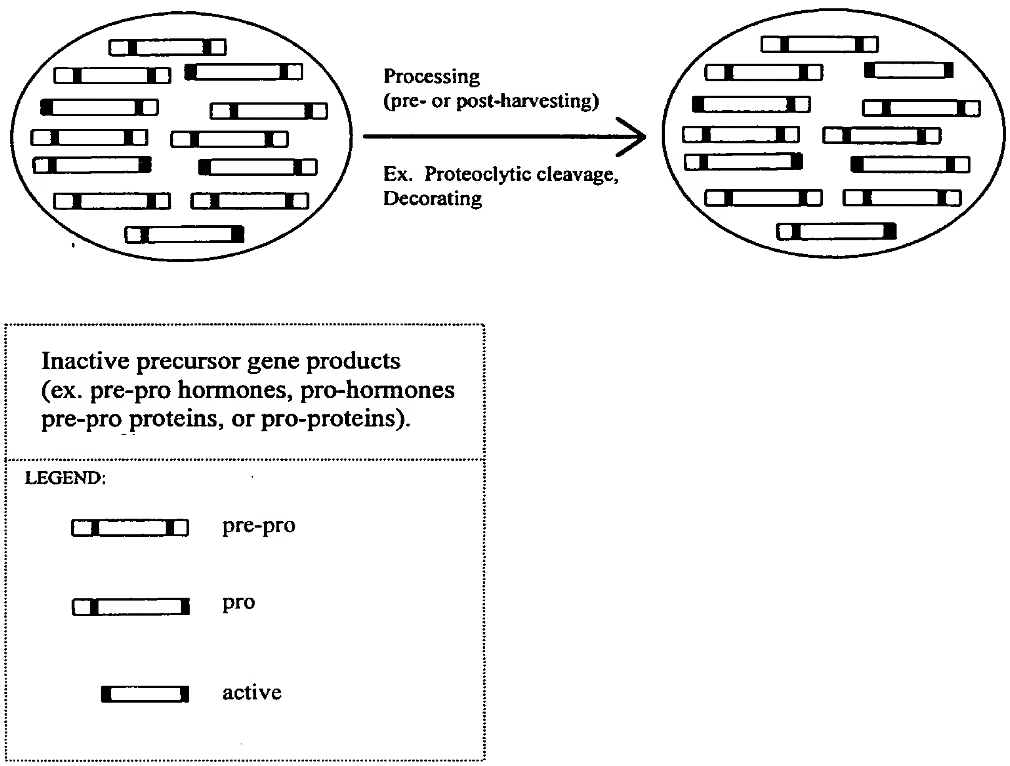


Figure 21. Starting population comprised of an organism strain to be subjected to improvement or evolution in order to produce a resultant population comprised of an improved organism strain that has a desired trait

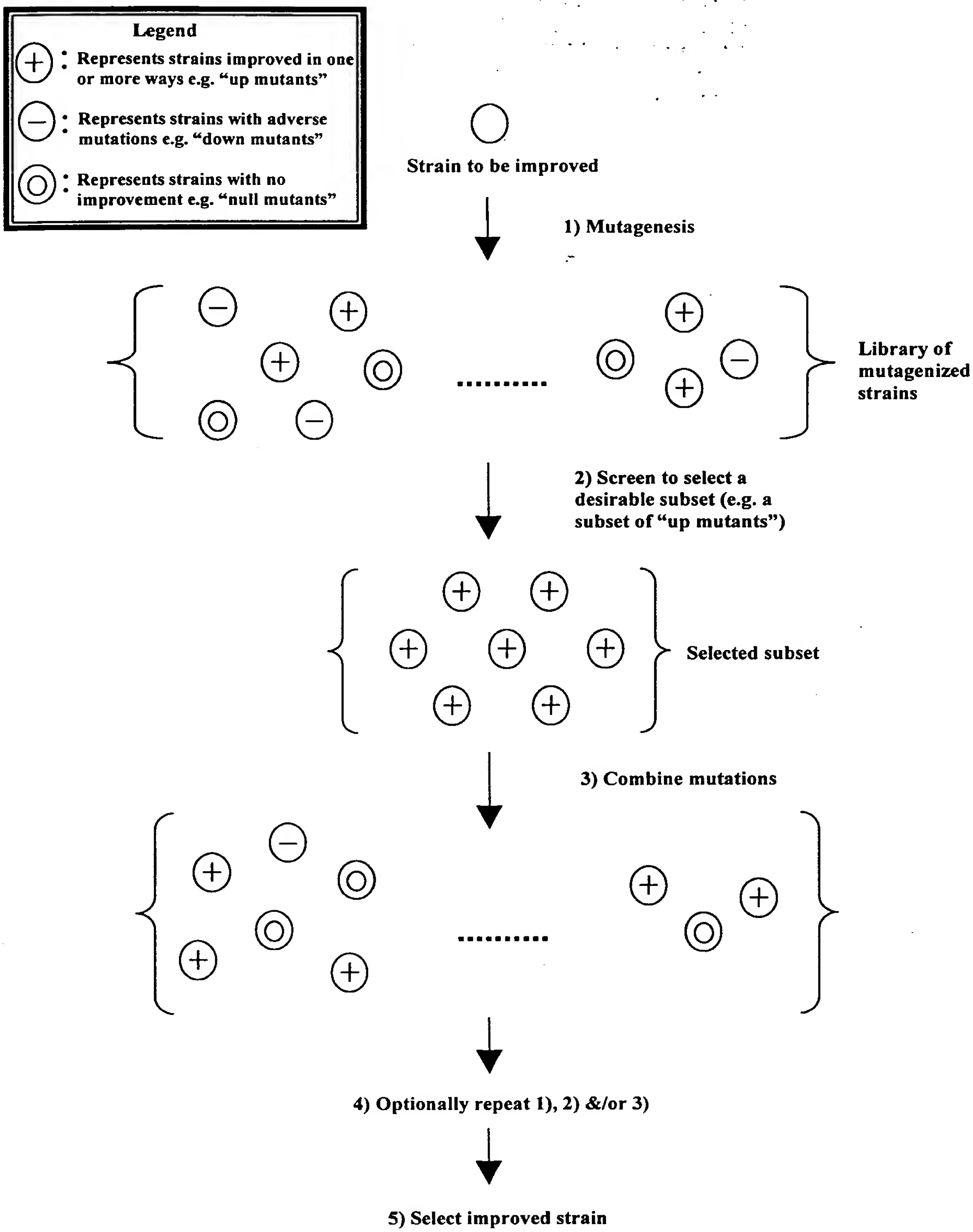


Figure 22. Starting population comprised of a genomic sequence to be subjected to improvement or evolution in order to produce a resultant population comprised of an improved genomic sequence that has a desired trait

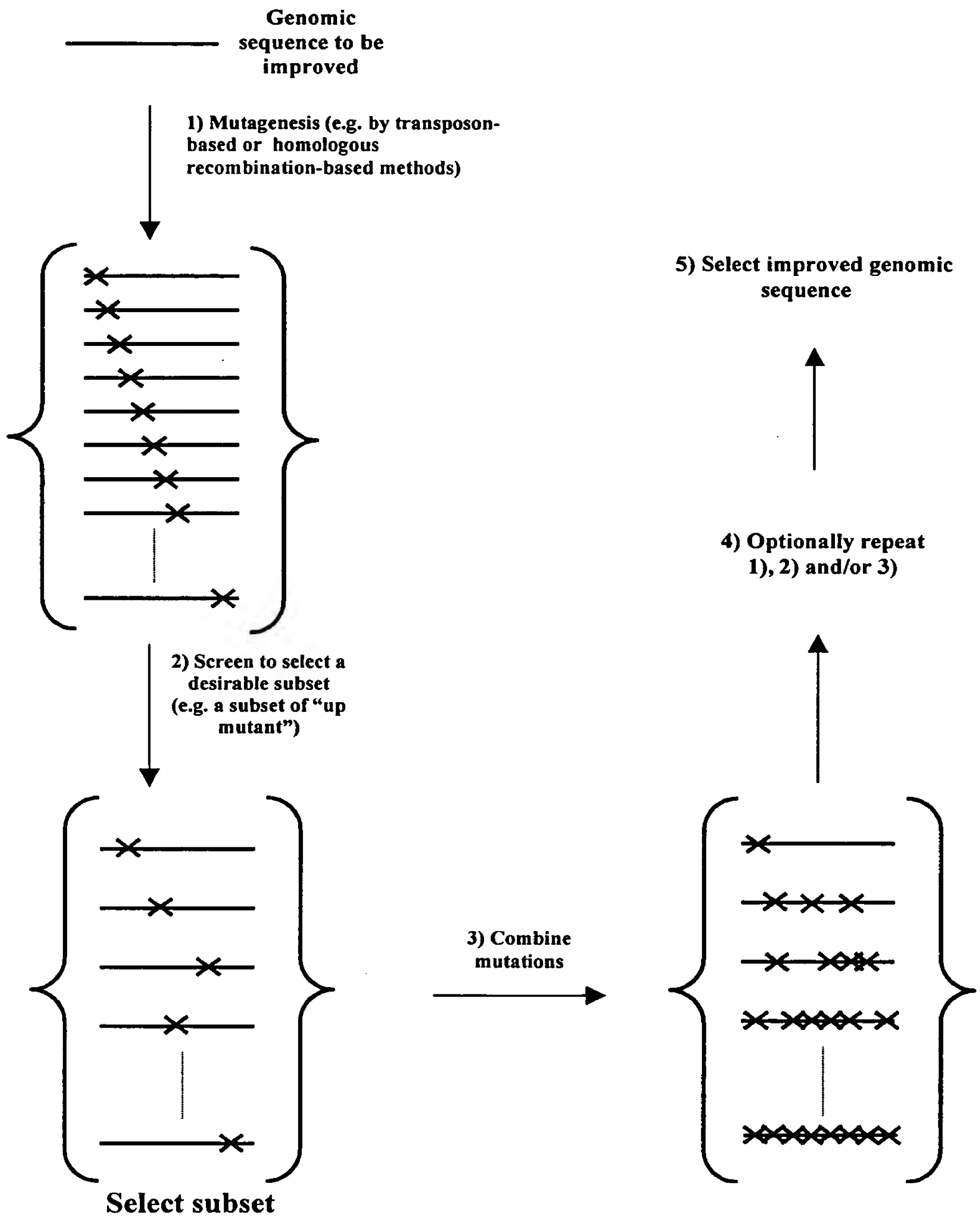


Fig. 23. Strain Improvement.

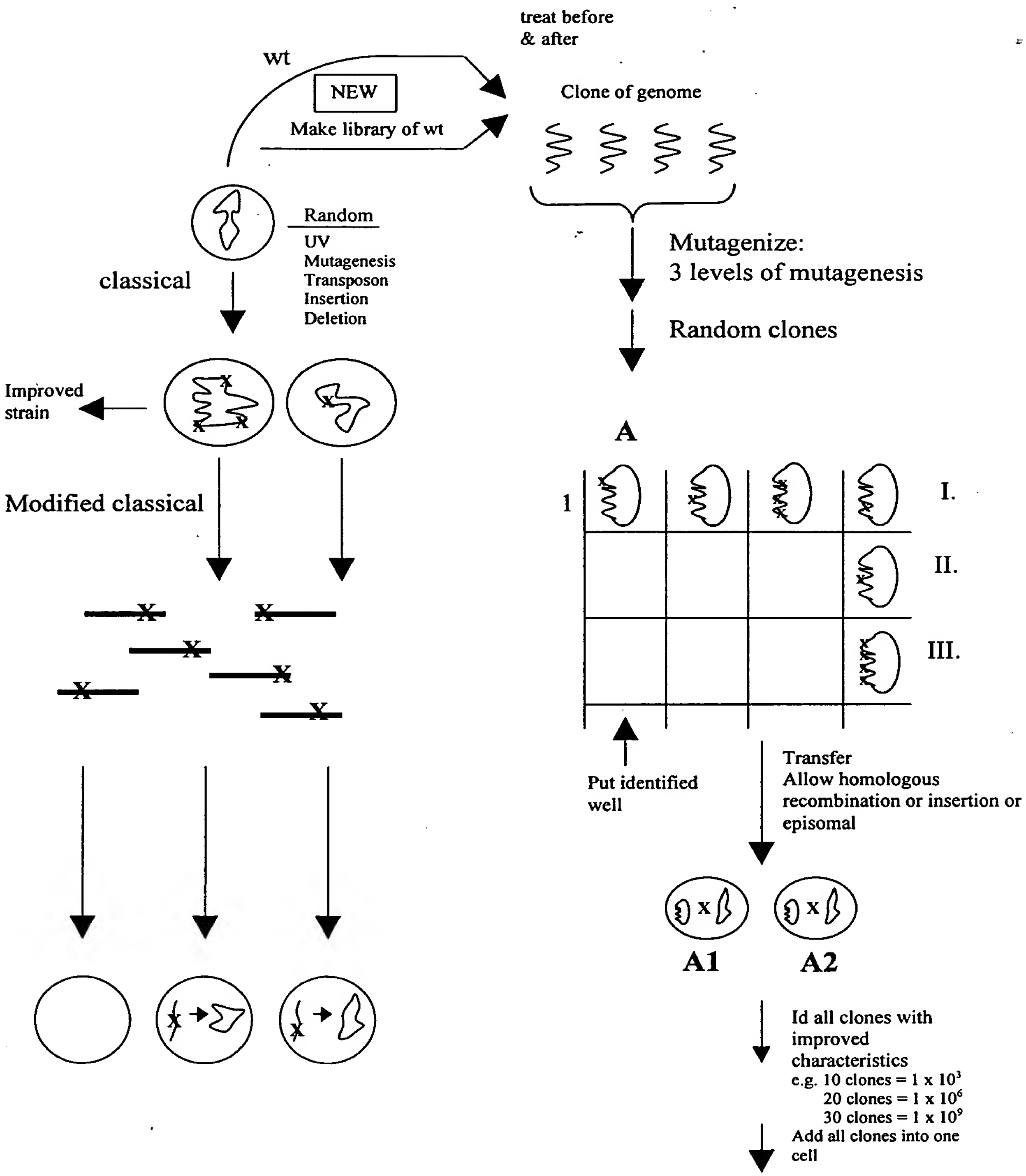


Fig. 24. Iterative Strain Improvement.